FINAL REPORT

Grant#: N00014-95-1-0584

PRINCIPAL INVESTIGATOR: Joel M. Weinberg M.D.

INSTITUTION: University of Michigan

GRANT TITLE: Relationship Between Glycine Cytoprotection and the Mitochondrial Permeability Transition During Hypoxic Cell Injury

AWARD PERIOD: 1 May 1995 - 30 April 1998

OBJECTIVE: To investigate the hypothesis that glycine and cyclosporine A (CsA) or other maneuvers that inhibit the Ca^{2+}-dependent, CsA-suppressible, mitochondrial membrane permeability transition (MPT) can strongly cooperate to protect hypoxic, renal proximal tubule cells via separate, but complementary effects to retard plasma membrane and inner mitochondrial membrane damage, respectively.

APPROACH: Freshly isolated preparations of proximal tubules from rabbit kidney are subjected to hypoxia alone or to hypoxia plus reoxygenation in the presence of glycine and modifiers of the MPT followed by measurements of in situ mitochondrial energization and inner membrane permeability properties, lytic plasma membrane damage, and the extent of recovery during reoxygenation of metabolic and transport function and structure.

ACCOMPLISHMENTS:

We initially demonstrated that tubules protected by glycine against lethal plasma membrane damage during hypoxia/reoxygenation develop a progressive deficit of mitochondrial ATP production, which becomes sufficiently severe at durations longer than 30 min. to result in cell killing if glycine is withdrawn during the reoxygenation period. If glycine is present during the reoxygenation period, acute cell killing does not occur, but there is a persistent, severe defect of ATP production, i.e. at the end of 60 min. of reoxygenation following 60 min. hypoxia, ATP levels are only 10-20% of time control values and increase only moderately when the tubules are supplemented with exogenous ATP to restore precursors for intracellular resynthesis of ATP. This lesion occurs irrespective of whether the pH during hypoxia is 7.4 or 6.9 and keeping the pH at 6.9 during reoxygenation does not prevent it. We showed that a combination of several agents known to inhibit development of the mitochondrial permeability transition, cyclosporine A, butacaine, and carnitine, ameliorates this deficit of mitochondrial function. Among them, cyclosporine A and butacaine were active when used individually (Publication #1).

We succeeded in measuring the full extent of cytosolic Ca^{2+} increase occurring in the glycine-protected tubules and found, surprisingly, that it can reach or exceed 100 µM, then recover during reoxygenation (Publication #2) and is compatible with long term viability (Publication #3). However, in our studies up to now this behavior appears to be dissociated from the mitochondrial functional change because total cell Ca^{2+} measurements do not indicate mitochondrial Ca^{2+} accumulation during hypoxia or reoxygenation and prevention of increased cytosolic Ca^{2+} by removal of medium Ca^{2+} does not

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	23 Dec 1998	Final, 1 May 1995 - 30 April 1998

4. TITLE AND SUBTITLE
Relationship Between Glycine Cytoprotection and the Mitochondrial Permeability Transition During Hypoxic Cell Injury

5. FUNDING NUMBERS
G N00014-95-1-0584
PE 61153N
PR RR04108

6. AUTHOR(S)

Joel M. Weinberg, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Michigan
1150 W. Medical Center Dr.
1560 MSRB II
Ann Arbor, MI 48109-0676

8. PERFORMING ORGANIZATION REPORT NUMBER

Not Applicable

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Office of Naval Research, Code 1141SB
800 N. Quincy Street
Arlington, VA 22217

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

Not Applicable

11. SUPPLEMENTARY NOTES

None

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The studies supported by this grant have shown that, during hypoxia/reoxygenation injury, mitochondria of kidney proximal tubule cells develop a profound functional deficit despite prevention of lethal plasma membrane damage by glycine. This is related to a persistent electron transport block at Site I. The mitochondria remain energized, but to a reduced degree. The lesion precedes development of the mitochondrial permeability transition and/or release of cytochrome c and is relatively stable. It can be prevented by mitochondrial permeability transition inhibitors and is even more potently prevented and reversed by substrates that contribute to anaerobic, intramitochondrial, substrate level phosphorylation or that can bypass, during reoxygenation, the block at Site I.

14. SUBJECT TERMS

Hypoxia, Ischemia, Kidney, tissue injury, Mitochondria, glycine

15. NUMBER OF PAGES
5

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
unclassified	unclassified	unclassified	SAR

affect the energetic deficit.

When we examined the ultrastructure of the tubules, we found that the mitochondria of controls had a normal "orthodox" configuration. Hypoxia rapidly induced a condensed configuration of the mitochondria lasting the full 60 min. During reoxygenation under conditions where function recovered, the mitochondria resumed an orthodox configuration. But, when function did not recover, they remained condensed. They did not swell as would be expected had a fully developed MPT occurred. As indicated by measurements of tetramethylrhodamine uptake, mitochondria in tubules examined by confocal microscopy maintained membrane potential ($\Delta\Psi$) during reoxygenation despite occurrence of the functional deficit, but the potential was substantially reduced. We quantitated this behavior using the dye, JC-1, which allows for ratios between red (energized) and green (deenergized) emissions and found that the red/green fluorescence ratios for the reoxygenated tubules were 44% of control values. In contrast, tubules that were completely de-energized with uncoupler were 5% of control values. Measurements of respiratory function with site specific substrates in digitonin-permeabilized tubules showed marked inhibition of the respiratory chain at Site I, moderate inhibition at Site II, and intact function at Site III. The preservation of Site III function indicates retention of cytochrome c. These observations provide evidence for a sustained electron transport defect that is associated with persistent mitochondrial dysfunction during hypoxia/reoxygenation without progression to a full MPT or cytochrome c release. That the MPT inhibitors are beneficial in this setting suggests that this may represent an incomplete form of the MPT as has been suggested in studies of isolated mitochondria, but we do not at this time have proof that this is the case (Publication #7).

Although the MPT inhibitors ameliorated the lesion, their effects were partial and it was necessary to have them present during both hypoxia and reoxygenation (Publication #1). The ability of substrates that can promote anaerobic substrate level phosphorylation in mitochondria to alleviate tissue injury has been known for several decades, but their mechanism of action has been controversial and this has limited further development and application of the concept. We tested whether substrate level phosphorylation in mitochondria could generate ATP to prevent or repair the mitochondrial lesion in the glycine-protected tubules. Tubules were incubated with their usual substrates or were supplemented with 4 mM each of α-ketoglutarate+aspartate (αKG/ASP), α-ketoglutarate +malate (αKG/MAL), or glutamate+malate during either the 60 min hypoxia, or the entire 60 min of reoxygenation, or for only the last 30 min of reoxygenation. Without supplemental substrates, cell ATP at the end of 60 min hypoxia plus 60 min reoxygenation was 12% of the control level. The substrate combinations during either hypoxia or reoxygenation significantly improved recovery of cell ATP three to four fold, normalized mitochondrial membrane potential, and restored an "orthodox" mitochondrial configuration. The benefit was appreciably greater than that provided by the MPT inhibitors in paired experiments. Of the three substrate combinations, αKG/MAL was strongest. It was beneficial at concentrations as low as 0.1 mM and was maximally effective at 1 mM. These substrates as well as fumarate and citrate were also active when provided individually, but less so than the combinations. Succinate, an aerobic substrate which bypasses Site I of the respiratory chain, was beneficial, but only during reoxygenation. Fructose, pyruvate, acetate, acetoacetate, and β-hydroxybutyrate had little or no effect. These

results indicate that provision of small amounts of ATP by substrate level phosphorylation or bypass of the electron transport defect at Site I plays a critical role in maintaining and recovering function of mitochondria during hypoxia/reoxygenation (Publication #8).

To directly assess the metabolic pathways involved in these protective effects, tubules were incubated with either [3-^{13}C] or [^{15}N]-aspartate±αKG (4 mM of each) during 60 min hypoxia or 60 min control oxygenated conditions with measurement by gas chromatography-mass spectroscopy of isotopomer enrichment and metabolite mass and by HPLC of amino acid levels. As compared to control tubules, hypoxic tubules incubated with ^{13}C-aspartate had decreased ^{13}C enrichment of citrate (0.45x), but increased enrichment of malate (1.33x) and succinate (6.04x) isotopomers, indicating reverse operation of the citric acid cycle. Addition of αKG resulted in isotopic dilution of ^{13}C-labeled metabolites while at the same time doubling both ^{13}C- and total succinate accumulation, which indicates simultaneous forward operation of the citric acid cycle as well as reciprocal promotion of αKG and aspartate metabolism during hypoxia by redox cycling of NAD. Cell ATP levels at the end of 60 min. hypoxia were increased from 0.16±0.02 to 0.27±0.02 nmol/mg protein by αKG/ASP ($p<0.01$), but were not affected by ASP alone. These data provide direct evidence for both anaerobic substrate level phosphorylation by metabolism of αKG and aspartate during hypoxia as well as succinate production that can further support recovery of mitochondrial function during reoxygenation (Publication #9).

Given the increased recognition that emerged during the funding period of the importance of apoptotic cell death and the discovery that cytochrome c release from mitochondria is a major mediator of that process, we have also further studied its expression in tubule cells as a basis for better understanding their injury responses. In Publication #4 we showed that DNA fragmentation by intranucleosomal cleavage, considered to be a hallmark of apoptosis, can also occur as an early event during necrotic cell death but appears to be triggered in that setting by serine proteases rather than cysteine proteases. In addition to suggesting a novel pathway of endonuclease activation during necrosis not involving participation of caspases, the data indicate that widely used techniques based on the occurrence of double stranded DNA breaks may not reliably distinguish between apoptosis and necrosis.

In Publication #5 we studied the regulation of apoptosis in cultured proximal tubule cells during hypoxia/reoxygenation. Unlike the freshly isolated tubules, apoptosis is the predominant mode of cell death in the cultured cells when they are protected by glycine from early lethal plasma membrane damage. We demonstrated that apoptosis under these conditions is mediated by the translocation of Bax to mitochondria which induces release of cytochrome c and caspase activation. The process is unidirectional insofar as inhibition of caspases prevents apoptotic nuclear damage, but does not affect the cytochrome c release. This is the first comprehensive description of the involvement of mitochondrial pathways leading to apoptosis in hypoxia/reoxygenation injury (Publication #6).

Finally, we have made progress elucidating the fundamental plasma membrane lesion that is prevented by glycine. In Publication #7 we showed using fluorescent dextrans and optical sectioning by confocal microscopy that this involves the formation of a progressively enlarging plasma membrane pore of molecular dimensions. Moreover, this glycine-sensitive "death channel" could alternatively be stabilized with a

homobifunctional, "nearest neighbor" crosslinking agent, indicating that its formation likely involves rearrangement of membrane proteins.

CONCLUSIONS: During hypoxia/reoxygenation injury mitochondria of kidney proximal tubule cells develop a profound functional deficit despite prevention of lethal plasma membrane damage by glycine. This is related to a persistent electron transport block at Site I. The mitochondria remain energized, but to a reduced degree. The lesion precedes development of the MPT and/or release of cytochrome c and is relatively stable. It can be prevented by MPT inhibitors and is even more potently prevented and reversed by substrates that contribute to anaerobic, intramitochondrial, substrate level phosphorylation or that can bypass, during reoxygenation, the block at Site I.

SIGNIFICANCE: Recognition of the critical role played by glycine in preventing lethal plasma membrane damage has provided insight into a novel, intrinsic cytoprotective mechanism expressed in diverse cells and new opportunities for studying mechanisms of cell injury in vitro under conditions more relevant to in vivo behavior. The identification of mitochondrial damage as a limiting factor despite the presence of glycine and its expression in glycine protected tubules in vitro has allowed elucidation of the mechanism of a very primary form of mitochondrial injury and the development of approaches for preventing and reversing it. These approaches deserve reassessment in whole animal models of forms of tissue injury that commonly occur during trauma. The observations provide new directions of study for further defining the contribution of mitochondrial damage to cellular dysfunction and techniques to alleviate it.

PUBLICATIONS AND ABSTRACTS (for total period of grant):

1. WEINBERG, J.M., N.F. Roeser, J.A. Davis, and M.A. Venkatachalam. Glycine-protected, hypoxic, proximal tubules develop severely compromised energetic function. Kidney Int. 52:140-151, 1997.

2. WEINBERG, J. M., J. A. Davis and M. A. Venkatachalam. Cytosolic free calcium increases to greater than 100 micromolar in ATP-depleted proximal tubules. J. Clin. Invest. 100:713-722, 1997.

3. Dong, Z., P. Saikumar, G.A. Griess, J. M. WEINBERG, and M.A. Venkatachalam. Intracellular Ca^{2+} thresholds that determine survival or death of energy deprived cells. Am. J. Pathol. 152:231-240, 1998.

4. Dong, Z., P. Saikumar, J. M. WEINBERG, and M.A. Venkatachalam. Internucleosomal DNA cleavage triggered by plasma membrane damage during necrotic cell death: involvement of serine but not cysteine proteases. Am. J. Pathol. 151:1205-1213, 1997.

5. Saikumar, P., Z. Dong, Y. Patel, K. Hall, U. Hopfer, J.M. WEINBERG, and M.A. Venkatachalam. Role of hypoxia induced Bax translocation and cytochrome c release in reoxygenation injury. Oncogene (in press)

6. Dong, Z, Y. Patel, P. Saikumar, J.M. WEINBERG, and M.A. Venkatachalam. Development of porous defects in plasma membranes of ATP-depleted Madin-Darby Canine Kidney Cells and its inhibition by glycine. Laboratory Investigation 78:657-668, 1998.

7. Venkatachalam, M.A., I. Nissim I, J.M. WEINBERG. Mechanism of mitochondrial dysfunction in proximal tubules during hypoxia/reoxygenation. J. Am. Soc. Nephrol. 9:589A, 1998 (abstract).

8. WEINBERG, J.M., M. A. Venkatachalam, N. F. Roeser, J. A. Davis, and I. Nissim. Prevention and reversal of mitochondrial dysfunction in proximal tubules during hypoxia /reoxygenation. J. Am. Soc. Nephrol. 9:591A, 1998 (abstract).

9. Nissim, I, M.A. Venkatachalam, N. F. Roeser, and J. M. WEINBERG. Direct evidence for protection of proximal tubule mitochondria during hypoxia/reoxygenation by anaerobic substrate level phosphorylation. J. Am. Soc. Nephrol. 9:582A, 1998 (abstract).